UNCLASSIFIED

AD NUMBER

AD815213

NEW LIMITATION CHANGE

TO

Approved for public release, distribution unlimited

FROM

Distribution authorized to U.S. Gov't. agencies and their contractors; Foreign Government Information; DEC 1964. Other requests shall be referred to the Army Biological Center, Attn: SMUFD, Fort Detrick, MD 21701.

AUTHORITY

SMUFD d/a ltr 14 Feb 1972

THIS PAGE IS UNCLASSIFIED

AD815213

THE HEMOLYTIC PROPERTIES OF DRIED PLAGUE BACILLI

TRANSLATION NO. 1249

December 1964

[redacted]

DDC
JUN 8 1967
E

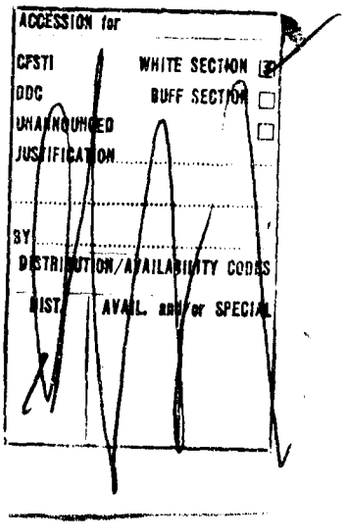
[illegible] LIED

This document is subject to special export controls and each transmittal to foreign governments or foreign nationals may be made only with prior approval of U. S. ARMY BIOLOGICAL CENTER Fort Detrick, Frederick, Maryland

DDC AVAILABILITY NOTICE

Qualified requestors may obtain copies of this document from DDC.

This publication has been translated from the open literature and is available to the general public. Non-DOD agencies may purchase this publication from Clearinghouse for Federal Scientific and Technical Information, U. S. Department of Commerce, Springfield, Va.



Technical Library Branch
Technical Information Division

Experimental Results

Hemolytic acitvity of lyophilized plague bacilli was manifest either immediately after drying, or only after 1-2 days to 1-2 months of storage in the refrigerator. In the latter case it gradually increased to a definite level, after which it remained unchanged for at least two years (the length of our observation of lyophilized preparations). In some, the hemolytic activity of lyophilized plague bacilli was 7-8 times higher than that of rinsed and aerated plague bacilli not subject to lyophilization (Tkachenko, 1961a).

The relationship between the dynamics of increment in hemolytic properties of lyophilized plague bacilli and the growing conditions of the original cultures before they were lyophilized could not be established. On the other hand, judging from the rates at which autolysis developed in the rinsed bacterial mass [See Note], we can assume that the more strongly pronounced are the autolytic processes in the mass before lyophilization, the earlier and more intensively does the hemolytic activity of rinsed plague bacilli after lyophilization appear. Even after brief storage of the original plague bacillus cultures, when their rinsing did not succeed in revealing appreciable signs of autolysis, the lyophilized preparations evidenced hemolytic activity, and during the storage of these preparations it often attained the same value as for distinct signs of autolysis. This last fact led to the thought that in and of itself the process of drying bacteria from the frozen state, promoting autolysis of cells, favors increase in the hemolytic activity of the lyophilized preparations during their storage period.

([Note]: In distinct autolysis rinsed bacterial mass became slimy and highly viscous.)

Therefore, apparently a definite parallel exists between the decay of plague bacillus cells and the liberation of a hemolytic factor from the cells.

Based on the intermittent relationship between the plague hemolysin and the structure of the bacterial cell, we attempted to find out precisely which components of these bacterial cells are related to the hemolytic activity of the plague bacillus. With this purpose in mind we investigated water-soluble toxic and water-insoluble fractions, and also the lipopolysaccharide of the plague bacillus in final concentrations of 1.2 mg/ml and 2.5 mg/ml.

According to the results of our experiments, not one of the water-soluble fractions (I, II, III, and IV) according to Walker, IA, IB, and II [See Note 1] according to Baker et al proved capable of inducing lysis of rinsed guinea pig and rabbit erythrocytes at 37° for three hours. The plague bacillus lipopolysaccharide was also devoid of hemolytic properties. Also these preparations proved to be hemolytically inactive in the presence of calcium ions (final dilution $2.5 \cdot 10^{-4}$ M and $3.25 \cdot 10^{-3}$ M) and magnesium ions (final dilution $3.25 \cdot 10^{-3}$ M), common lipase activators, including C-lecithinase of Bac. perfringens (MacFarlane and Knight, 1941) [See Note 2].

([Note]: The LD_{50} for white mice (according to Read and Mench) equalled about 5-10 grams.)

([Note]: Ordinary and buffered (0.033 M veronal buffer, pH 7.5) physiological saline solutions were used as media.)

In parallel experiments a preparation of the Bac. perfringens toxin induced 50% lysis of rinsed guinea pig and rabbit erythrocytes during half an hour with and without the presence of calcium and magnesium ions.

Water-insoluble residues of plague bacillus proved hemolytically active when fractionated according to the Walker method as modified by I. V. Domaradskiy and following the method of Baker et al.

The quantitative estimate of hemolytic activity of dried water-insoluble residues was made from the mean values of the yield of the latter upon fractionation (Table 1) and recalculation of hemolytic activity for the integral bacterial cells was made.

Hemolytic activity of water-insoluble residues at the final concentration, which was $\frac{1}{4}$ that of the original lyophilized bacteria, was 10-12 and even 20 times higher, but the hemolytic activity of rinsed and aerated plague bacilli not undergoing lyophilization was 60-130 times greater [than the starting lyophilized bacteria].

In some cases hemolytic activity of just prepared water-insoluble residues, as true also in the case of lyophilized plague bacillus cultures, somewhat increased during storage, but this increase was relatively rapid and short-lived.

Table 1

Mean Yield of Water-Soluble Residues upon Fractionation of Plague Bacillus According to the Method of Baker et al, and According to the Walker Method as Modified by I. V. Domaradskiy et al

(a) Фракционирование чумных бактерий	(b) Вакцинные штаммы		
	1	17	EB
По способу Бейкера и др. (c)	40,0%	42,2%	45,5%
По способу Уокера в модификации И. В. Домарадского и др. (d)	35,3%	35,6%	27,6%

[Legend:] a) fractionation of plague bacilli; b) vaccine strains; c) according to the method of Baker et al.; d) according to the Walker method as modified by I. V. Domaradskiy.

Lyophilized plague bacillus preparations and their water-insoluble residues proved to be hemolytically more active than those acetone-dried. This difference is especially appreciable in the comparison made of hemolytic activity of water-insoluble plague bacillus residues obtained upon lyophilization with those obtained following acetone drying (Table 2).

Graphically, the course of the hemolysis reaction with time [See note] in the testing of lyophilized plague bacilli and their water-insoluble residues, as true also for rinsed aerated bacteria not undergoing lyophilization, takes the form of an S-shaped curve. But this curve is characterized by a steeper ascent in the case of the lyophilized bacteria and especially in the case of their lyophilized water-insoluble residues.

([Note]: In the construction of the graph extinction values obtained from sample colorimetry were plotted on the ordinate axis, and on the abscissae axis -- incubation rates of each of the samples (in minutes).)

A definite relationship was found to hold between the concentration of the preparation tested and the time 50% hemolysis occurred. Under our experimental conditions the time of

Table 2

Hemolytic Activity of Rinsed Plague Bacilli and Their Water-Insoluble Residues According to Baker et al Following Lyophilization and Following Acetone-Drying

(a) Штамм	(b) Наименование и № препарата	(c) Способ сушки	(d) Гемолитическая активность в условных единицах по данным испытаний	
			(e) сразу после сушки	(f) в последующих опытах
1	Водонерастворимый остаток № 1 а (g)	лиофилизация (m)	380,6	400,0 (через 21 сутки) (p)
.	.	обработка ацетоном (n)	32,3	33,2 (через 21 сутки) (p)
17	Отмытые бактерии (h) № 2	лиофилизация (m)	0	25,6 (через 32 суток) (q)
.	.	обработка ацетоном (n)	0	10,8 (через 32 суток) (q)
.	Водонерастворимый остаток № 2 а (i)	лиофилизация (m)	350,0	417,6 (через 11 суток) (s)
.	.	обработка ацетоном (n)	31,5	31,6 (через 11 суток) (s)
.	Отмытые бактерии (j) № 3	лиофилизация (m)	не испытывалась (o)	25,2 (спустя 1 год) (r)
.	.	обработка ацетоном (n)	.	14,0 (спустя 1 год) (r)
.	Отмытые бактерии № 4 (k)	лиофилизация (m)	.	25,2 (спустя 1 год) (r)
.	.	обработка ацетоном (n)	.	11,2 (спустя 1 год) (r)
EB	Отмытые бактерии № 5 (l)	лиофилизация (m)	0	25,2 (через 29 суток) (t)
.	.	обработка ацетоном (n)	0	15,2 (через 32 суток) (q)

[Legend:] a) strain; b) name and number of preparation: c) drying method; d) hemolytic activity in arbitrary units from testing data; e) immediately after drying; f) in subsequent experiments; g) water-insoluble residue No 1 a; h) rinsed bacteria No 2; i) water-insoluble residue No 2 a; j) rinsed bacteria No 3; k) rinsed bacteria No 4; l) rinsed bacteria No 5; m) lyophilization; n) acetone treatment; o) not tested; p) (in 21 days); q) (in 32 days); r) (in one year); s) (in 11 days); t) (in 29 days).

50% hemolysis remained unchanged when the concentration exceeded the final value of 1.25 mg/ml for lyophilized plague bacillus culture and 0.312 mg/ml for its water-insoluble residue; accordingly, the concentration indicated were used in subsequent experiments.

Hemolytic activity of lyophilized plague bacilli and their water-insoluble residues remained essentially unchanged after preliminary boiling for three hours. Hemolytic properties of these preparations were similar to those of rinsed aerated but nonlyophilized plague bacilli and in their capacity to cause lysis of rinsed guinea pig erythrocytes at 4°. However, lyophilized bacteria proved to be more active than nonlyophilized by approximately fourfold, and lyophilized water-insoluble residues -- by approximately 50-60 times.

Hemolytic activity of lyophilized preparations, true also for rinsed aerated but nonlyophilized plague bacilli, is suppressed by protein. Native sheep, equine, and guinea pig sera suppress hemolytic activity of lyophilized plague bacilli and their water-insoluble residues according to Baker et al by approximately the same extent. Also demonstrating an inhibiting effect, although less pronounced, is lyophilized purified gamma-globulin of equine serum, both normal and anti-plague, where the inhibiting strenth of latter is completely identical. This fact indicates that the inhibiting effect of protein on the activity of plague hemolysin is evidently devoid of a specific character. Egg albumin proved to have a still less pronounced inhibiting effect on the activity of plague hemolysin. All the protein preparations tested in our experiments were used at a final concentration of 0.75% (based on the protein, determined refractometrically) per sample.

Finally, like the hemolytic activity of rinsed and aerated plague bacilli the hemolytic activity of lyophilized bacilli and their water-insoluble residues was inhibited by adding calcium ions. Thus, for example, calcium ions (in a final concentration of $2.54 \cdot 10^{-4}$ M) reduced hemolytic activity of lyophilized water-insoluble residues of plague bacilli of strains 17 and EB according to Baker et al by approximately 3-3.2 times. The hemolytic activity of the original lyophilized plague bacillus cultures were inhibited still more sharply.

It was found in addition that the hemolytic activity of lyophilized preparations is inhibited also, although to a lesser extent, by magnesium ions (in a final concentration of

$3.2 \cdot 10^{-3}$ M). Cholesterol added to a final concentration of 0.29% also inhibits hemolysis caused by lyophilized plague bacillus preparations [See Note].

([Note]: The cholesterol was emulsified in an 0.1% sunflower oil emulsion. This cholesterol emulsion also failed to induce hymolysis.)

The hemolytic activity of lyophilized cultures and of the derived water-insoluble plague bacillus residues, in contrast to the activity of rinsed aerated cultures, proved to be dependent on the pH value of the medium (0.16 M phosphate buffer, pH 5.65, 7.21, and7.73; 0.16 M phosphate-citrate buffer, pH 5.62, 7.01, and 7.63): at pH < 7.0 the hemolytic activity was less than at pH > 7.0. However, the relationship observed has some peculiarities for each of the preparations tested. For example, the hemolytic activity of one of the lyophilized plague bacillus cultures of strain EB at pH = 5.65 was reduced by 30%, but increased by 40% at pH = 7.73 compared to its value at pH = 7.0. The hemolytic activity of lyophilized water-insoluble residue obtained from this culture, according to Baker et al, was reduced by 80% at pH = 5.65, but at pH = 7.73 it was augmented by 120%. The hemolytic activity of lyophilized water-insoluble plague bacillus residue varied very substantially upon shifts in pH of the medium on the acidic or alkaline side, apart from dependence on the length of storage of the preparations tested. Hemolytic properties of the original lyophilized bacillus cultures as a function of medium pH prevailed only in the testing of preparations stored for more than a year.

Change in hemolytic activity of lyophilized plague bacilli and especially of their lyophilized water-insoluble residues with change in pH of the medium in inversely proportional to the final concentration of these preparations in experiment; when the concentration was increased the difference of its hemolytic activity in the acidic and in the alkaline medium became less pronounced.

Finally, in contrast to rinsed and aerated plague bacilli, lyophilized water-insoluble residues, according to Baker et al, proved capable of lyzing rinsed human erythrocytes, in terms of sensitivity to hemolytic action of lyophilized plague bacillus cultures and of their water-insoluble residues rinsed animal and human erythrocytes were ranked in the following decreasing order: guinea pig - horse - rabbit - man - sheep.

Water-insoluble residues also with respect to rinsed erythrocytes of other animals and man were tens of times more hemolytically active than lyophilized plague bacillus cultures of the strains 17 and EB.

According to the data of Ye. M. Gubarev and N. N. Ivanovskiy (1958) et al, hemolysis induced by plague bacillus can be caused by volatile bases forming in the decomposition of bacterial cells, first of all -- ammonia. Ammonia is capable of forming a base and inducing typical alkaline hemolysis by generating in the medium an excess of hydroxyl ions. In addition, ammonium ions in all probability are not devoid of hemolytic properties. Thus, according to the data of Bell and Krantz (1959), ammonium chloride in a concentration close to the isotonic (0.15 M) lyzes human erythrocytes, while this hemolysis is suppressed in equal measure by chlorides of several alkali metals, such as lithium, potassium, etc. We also know that alkaline hemolysis is inhibited by several sugars (Morita, 1959).

In our experiments hemolytic activity of ammonia used in the ammonium hydroxide form, diluted in isotonic solution (0.16 M) of glucose, saccharose, and lactose to a final concentration of 0.007 M, was almost 50% less compared to the activity of ammonia diluted in physiological saline solution under otherwise equal conditions. Lyophilized rinsed aerated plague bacilli and their water-insoluble residues, having been suspended together with rinsed guinea pig erythrocytes both in a physiological saline solution as well as in isotonic solutions of the above listed sugars, evidenced almost identical hemolytic properties. In addition, some decrease in hemolytic activity was noted in the presence of the sugars used for lyophilized unrinsed plague bacilli (dry live EB vaccine) under otherwise equal conditions.

Further, in our experiments ammonium chloride diluted in an isotonic solution of glucose to a final concentration of 0.16 M, proved capable of inducing the lysis of rinsed guinea pig erythrocytes. Cations of the alkali metals Li and K, used in the chloride form in the final concentration of 0.16 M, reduced by almost twofold the activity of ammonium chloride and had completely no effect on the activity of the lyophilized rinsed aerated plague bacilli and of their water-insoluble residues, resulting in some decrease in activity only in lyophilized unrinsed plague bacilli.

The experiments quite convincingly demonstrated that hemolysis induced by plague bacillus and involved with the

decay products of bacterial cells is not likely to be caused by the activity of volatile organic bases, including ammonia; at least, the latter has altogether no determining effect on the hemolytic properties of rinsed plague bacilli and of their water-insoluble residues. The activity of volatile bases and ammonia can determine to some extent the hemolytic properties of plague bacilli only when raised in blood-containing media, which we noted earlier in a description of the lysis of canine erythrocytes (Tkachenko, 1961, unpublished data) or in the testing of unrinsed plague bacilli in the experiments described above.

Discussion

The results of the studies showed that despite the data of G. D. Belonovksiy (1904), S. I. Zaplatine (1959), et al hemolytic activity of plague bacilli has not relationship to its toxin. Our data is in complete agreement with the assertion of Delaunay and Lebrun (1947) et al on the lack of hemolytic properties of toxins of gram-negative bacteria.

Noting that hemolytic properties are shown by toxic plague bacilli fractions [See Note], S. I. Zaplatine (1959) described the lecithinasic activity of these fractions and, coordinating their hemolytic activity with the lecithinasic, concluded that plague toxin is analogous to alpha-toxin of Bac. perfr gens.

([Note]: In our investigations we could not obtain and test fractions studied by S. I. Zaplatina (1959), since we have not found a detailed description of the original method of deriving these fractions, although S. I. Zaplatina made a reference to another study (Gubarev et al, 1955). In the latter it is in fact stated that the method is based on "fractionation of proteins soluble in weak salt solutions... by precipitation with different concentrations of ammonium sulfate," which incidentally lies at the basis of, for example, the method of Baker et al which we have used.)

Plague bacillus and its fractions are devoid in general of lipolytic properties (Rykova and Domaradskiy, 1961, unpublished data). Essel'man and Liu (1961) have shown that growing plague bacillus cultures have no lecithinasic activity. Moreover, lecithinasic activity is absent in hemolytically active residues (Domaradskiy et al, 1962). According to the data of MacFarland and Knight (1941), MacFarland et al (1941), lecithinasic activity and hemolytic activity of the Bac. perfringens toxin invariably accompany each other. Finally,

toxic plague bacillus fractions not evidencing hemolytic properties under the conditions of our experiments have proved to also lack lecithinasic activity (Domaradskiy et al, 1962).

Thus, plague bacillus does not exhibit lecithinasic activity, and its hemolytic properties by their nature are wholly distinct from the hemolytic properties of the Bac. perfringens alpha-toxin.

The activity of plague hemolysin with respect to the erythrocytes of many animals and man, its thermal stability, the inhibiting effect of protein, cholesterol, ions of calcium, magnesium, and hydrogen, and the activating effect of hydroxyl ions -- all these features of plague bacillus hemolytic activity compel the assumption that the activity of higher fatty acids underlie its character.

Conclusions

1. Lyophilized plague bacillus cultures are characterized by the presence of relatively weakly pronounced hemolytic properties, and the hemolytic activity of lyophilized plague bacillis is in direct dependence on the extent of their autolysis.

2. When lyophilized plague bacilli are fractionated their toxic fractions are devoid of hemolytic properties; the water-insoluble plague bacillus residues not showing lecithinasic activity are hemolytically active.

3. Hemolytic activity of lyophilized water-insoluable residues is ten times greater than the activity of the original lyophilized plague bacilli.

4. The hemolytic activity of water-insoluble residues are analogous to the activity of rinsed and aerated plague bacilli both before and after their lyophilization with respect to several properties (thermal stability, extent of activity toward erythrocytes of various animals, inhibiting action of protein, calcium ions, etc.).

5. Distinguishing features of the hemolytic activity of lyophilized plague bacilli and their water-insoluble residues is the constancy of the activity, its inhibition by excess H ions, and activation by excess OH ions, the capacity to lyse human erythrocytes.

6. Hemolytic properties of lyophilized plague bacilli and their water-insoluble residues are inhibited in the presence of magnesium ions and cholesterol.

7. Hemolytic activity of rinsed plague bacilli and their water-insoluble residues following aceton drying is considerably less than following lyophilization of the plague bacilli.

LITERATURE

Belonovskiy, D. D., "Hemolysins of Plague Toxins," Arkhiv biologicheskikh nauk (Archives of Biological Sciences), 1904, Vol 10, No 4.

Gubarev, Ye. M., Zaplatina, S. I., and Konnova, A. M., "Fractionating Studies of Protein Components of the Plague Bacillus," Mezhinstitutskaya nauchnaya konferentsiya po voprosam mikrobiologii, immunologii, laboratornoy diagnostiki i terapii osobo opasnykh infektsiy i proizvodstvu bakteriynykh preparatov (Interinstitute Scientific Conference on Problems of Microbiology, Immunology, Laboratory Diagnosis and Therapy of Expecially Dangerous Infections, and the Production of Bacterial Preparations), Ministry of Public Health USSR, "Mikrob" Institute, Saratov, 1955.

Domaradskiy, I. V. Yaromyuk, G. A., and Kalmykova, A. P. "New Method of Obtaining Toxic, Immunogenic, and Fibrinolytically Active Plague Bacillus Fractions," Doklady Irkutskogo protivochumnogo instituta (Reports of the Irkutsk Anti-Plague Institute), No 2, Chita, 1961.

Druzhinina, K. V. and Kritsman, M. G. "Lecithinase of Animal Tissues," Biokhimiya (Biochemistry), 1952, Vol 17. No 1.

Zaplatina, S. I., "Study of the Enzymatic Character of Plague Bacillus toxins," Trudy Rostovskogo-na-Donu gosudarstvennogo nauchno-issledovatel'skogo protizochumnogo instituta (Works of the Rostov-na-Don State Scientific-Research Anti-Plague Institute), Vol 15, No 1, Rostov-na-Don, 1959.

Tkachenko, V. V., "Hemolytic Properties of Rinsed Plague Bacillus Cultures," Doklady Irkutskogo protivochumnogo instituta, No 1, Ulan-Ude, 1961a.

Tkachenko, V. V., "Method of Estimating Hemolysis Reaction by Using the FEK-M Photoelectric-Colorimeter," Doklady Irkutskogo protivochumnogo instituta, No 1, Ulan-Ude, 1961b.

Baker, E. E., Sommer, H., Foster, L. E., Meyer, E., and Meyer, K. F., "Studies of Immunication against plague 1. The Isolation of the Soluble Antigen of P. Pestis." J. Immunol., 1952, Vol 62, No 2.

Bell, F. K., Krantz, J. C. Jr., "Effect of Ions of Alkali Metals on Hemolytic Action of Ammonium Chloride," Arch. Internat. Pharm., 1959, Vol 121, No 1-2.

Davies, D. A. L., "Smooth and Rough Antigens of Past. Pseudotuberculosis," J. Gen. Microbiol., 1959, Vol 18, No 1.

Delaunay, A. and Lebrun, J., "Le mode d'action des endotoxines bacteriennes. 1. Introduction a une etude generale," Ann Insti. Pasteur, 1947, Vol 73, No 6.

Esselman, M. T. and Liu, P. V., "Lecithinase Production by Gram Negative Bacteria," J. Bacteriol., 1961, Vol 81, No 6.

MacFarlane, M. G. and Knight, B. C. J. G., "The Biochemistry of Bacterial Toxins. 1. The Lecithinase Activity of Cl. Welchii Toxins," Biochem. J., 1941, Vol 35.

MacFarlane, M. G., Oakley, C. L. and Anderson, C. G., "Hemolysis and Production of Opalescence in Serum and Lecithovitellin by the - Toxin of Cl. Welchii," J. Path. and Bact., 1941, Vol 52.

Morita, K. I., "Inhibiting Action of Sugars on Alkaline Hemolysis, "(in Japanese), Shikoku Acta Med., 1959, Vol 14.

Walker, J. "A Method for the Isolation of Toxic and Immunizing Fractions from Bacterial of the Salmonella Group." Biochem. J., 1940, Vol 34, No 3.

- END -